STUDIES IN ARTIFICIAL PARTHENOGENESIS. III. CORTICAL CHANGE AND THE INITIATION OF MATURATION IN THE EGG OF CUMINGIA.[1]

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This study is a record of experiments performed during the summer of 1916 at the Woods Hole Marine Biological Laboratory.

As Morgan pointed out in 1910, the egg of the lamellibranch *Cumingia* is very suitable for study. Like most other eggs it is still immature when shed into the sea-water. Although the first maturation spindle has formed, no polar bodies are thrown off unless the egg is fertilized or treated with the proper reagents. Doubtless some change is necessary before the egg can throw off polar bodies and begin its development.

An effort has been made to determine the nature of this change. Many diverse reagents cause the egg to mature. Although all of these reagents do not occasion the same morphological transformations nevertheless all of them agree in having one specific physical effect on the egg. All release the egg cytoplasm from the restraint of a rigid enveloping membrane. The immature unfertilized egg is surrounded by a stiff vitelline membrane which presses tightly in on it and effectively prevents the throwing off of polar bodies. It is only when the egg is released from this restraint that maturation can proceed.

Physical Make-up of the Egg.

As in *Arbacia*, the *Cumingia* egg is a mass of fluid protoplasm, surrounded by a rigid membrane. Only a few turns of the centrifuge are sufficient to throw to opposite poles of the egg the substances suspended in the cytoplasm. To one pole pass the presumably lighter oil globules, to the opposite pole the heavier pigment. But these suspended particles can go no farther than the poles, for there they are stopped by the vitelline membrane which surrounds the egg. This is a stiff structure and is easily

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visible under higher power. It is about one micron in thickness. The vitelline membrane must be thought of as the plasma membrane of the egg. As I have pointed out before, students of cellular mechanics have been blind to the fact that egg cells are provided with visible membranes which are plasma-membranes. They have often insisted that no one has ever seen a plasma-membrane. It is easy to prove that the vitelline mem-membrane of the *Cumingia* egg governs osmotic intercourse and is therefore a plasma-membrane.

When *Cumingia* eggs are put into hypertonic solutions they shrink only very slightly. The stiffness of the vitelline plasma-membrane prevents a marked shrinkage. Moreover, a weakly hypertonic solution produces just as much shrinkage as a strong one provided that it does not alter the membrane. On the other hand if the hypertonic solution makes the membrane less rigid by causing it to swell, then the egg shrinks to a great extent. Facts such as these can be interpreted only on the assumption of a stiff plasma-membrane. That this is the vitelline membrane is certain, for there is no other membrane in the vicinity. Certainly there is none inside the vitelline membrane, for when the egg has been left for some time in a hypertonic solution of considerable strength then the coagulated cytoplasm shrinks away from the vitelline membrane and presents a rough uneven surface. Under these conditions it is obviously not surrounded by any membrane.

As to the chemical nature of the plasma-membrane it is essentially protein. It swells in dilute acids or alkalies, and in sodium chloride or sodium iodide solutions. Moreover it does not contain any large admixture of lipoid as can be shown by testing it with Scharlach R solution.

Like many other marine eggs the Cumingia egg is surrounded by a diffuse jelly of the same refractive index as sea-water and therefore invisible. It can easily be demonstrated by various vital stains (*e.g.*, Nile blue sulphate) or by India ink suspensions. This jelly has no apparent effect on the early developmental phenomena. If the eggs are shaken a few times in a test-tube, they are deprived of their jelly. Such eggs react in the same way as those with jelly intact.

As in *Arbacia* the *Cumingia* vitelline membrane is capable of two kinds of cortical change. Both membrane elevation and membrane swelling can occur. If the egg is placed in solutions of low surface tension the tension of the membrane is lowered and it rapidly lifts away from the egg. No doubt the explanation of this process is the same as that I have offered for the similar process in the sea-urchin egg. But in *Cumingia* membrane elevation is not the normal cortical change. When eggs are inseminated the membrane does not become elevated, it becomes swollen.

Treatment of eggs with a reagent which causes either membrane elevation or membrane swelling will result in a throwing off of polar bodies. Thus just as in the sea-urchin egg either swelling or elevation provides the necessary cortical change, although in this egg it is swelling and not elevation which is normal. Finally there is a third way in which maturation can be initiated. This consists in the removal of the membrane.

I shall now proceed to the experimental data considering first membrane elevation, second membrane swelling, and third the removal of the membrane.

Membrane Elevation.

Any substance which markedly lowers the surface tension of sea-water is effective in producing membrane elevation. But if elevation is to be followed by maturation only certain concentrations and certain lengths of exposure can be used in each case. Too high a concentration or too long an exposure generally leads to coagulation. Frequently an over-exposure produces a rupture of the egg membrane, the protoplasm then flows out to form an exovate. Such eggs soon disintegrate.

Numerous reagents were used to produce a lowering of surface tension. Of course the number of successful reagents could have been increased many fold. Thus many of the higher alcohols no doubt act in the same way as the amyl alcohol which I used.

In the experiments the same general proceedure was always employed. In every case, the eggs of only a single female were used. Owing to the fact that the number of eggs obtainable from a single female is comparatively small, I found it best to

perform all of the experiments in low Stender dishes (containing about 50 cc.) instead of in larger fingerbowls. These dishes were very convenient, for they could without any trouble be put on the stage of the microscope. One Stender dish was made to contain the desired reagent and then the eggs were pipetted into it. After varying lengths of exposure the eggs were transferred from the reagent to fresh sea-water also contained in Stender dishes. Then after twenty minutes or more had elapsed the treated eggs were examined.

As the polar bodies of Cumingia are unusually large it is possible to count the percentage of matured eggs under low power and without removing the eggs to a slide. Counts were usually made in this way. Obviously it is not possible to see the polar bodies if the egg is lying with the animal pole down. Hence the per cent. recorded is always too low. Even if all the eggs had polar bodies not many more than fifty per cent. would show them. If the eggs are turned about this difficulty can of course be obviated, but turning is a tedious process and was only occasionally resorted to. Thus the *counts recorded represent minima* and are really only about half as high as they should be. Counts are given in the form of fractions, in which the denominator represents the total number of eggs counted the numerator the number of eggs with polar bodies. For example the fraction 10/50 would indicate that out of 50 eggs counted 10 showed a polar body.

In making up per cent. solutions of volatile liquids, it was found convenient to use 100 c.c. measuring flasks. Thus if a 2 per cent. ether solution was desired 2 c.c. of ether was placed in a 100 c.c. measuring flask and sea-water was added until the solution reached the 100 c.c. mark. Owing to the diminution in volume on mixing the two liquids such a solution is not exactly a 2 per cent. one. However, after the above method of procedure it is easier to calculate the molecular concentration.

The accompanying table gives the results of experiments with eleven substances which lower surface tension. For each substance used it was necessary first to determine the proper concentration. Sometimes this was a simple matter. Thus for saponin almost any concentration is successful. But usually only a narrow range of concentrations will produce the desired

effect. If the reagent is just a little too strong it can not be effectively employed. In many cases only a certain length of exposure is suitable. Too long exposures generally produce exovates. Formation of exovates is represented by the symbol "e" in the table.

With ether and chloroform it is particularly difficult to obtain maturation. In both instances I at first despaired of success. A 3 per cent. solution of ether in sea-water did not produce maturation. With such a solution exovates generally appeared. On the other hand 2.5 per cent. ether had no observable effect on the eggs. With a concentration intermediate between 2.5 per cent. and 3 per cent. success was attained. In the table are given the figures for a representative experiment with ether.

In the case of chloroform the range of effective solutions is even narrower. In making up chloroform solutions very small quantities of the liquid had to be measured out. This was done by counting the drops from a small pipette which was calibrated for the purpose. About 60 drops from this pipette constituted 1 c.c. By placing 8 such drops into a 100 c.c. measuring flask and diluting to the mark a 0.13 per cent. solution was obtained. This solution produced but little effect upon the eggs. Exposures of 3–5 minutes showed only 1–2 per cent. of polar body formation. Thus a 0.13 per cent. solution was apparently too weak. On the other hand, a 0.17 per cent. solution proved too strong. Such a solution was prepared by diluting 15 drops of chloroform (from the pipette mentioned above) up to 100 c.c. with sea-water. A 0.25 per cent. solution was thus obtained and this was then diluted to 0.17 per cent. by adding 5 c.c. of sea-water to 10 c.c. of the solution. The resultant 0.17 per cent. solution was found to be too strong, for it produced exovates. Moreover viscosity tests with the centrifuge showed that it coagulated the egg cytoplasm. Although neither 0.13 per cent. nor 0.17 per cent. chloroform produced maturation, a concentration slightly under 0.17 per cent. did. This solution was made by diluting 10 drops from the pipette up to 100 c.c. Such a solution would ordinarily give 0.17 per cent., but one particular solution was made on an extremely hot day (when the room temperature was 27°). No doubt the drops from the pipette were smaller, owing to the

lower surface tension. Hence the concentration which resulted was slightly under 0.17 per cent. In the table I have referred to this solution as "0.16 per cent." As the figures show, it proved highly effective.

TABLE I.

POLAR BODY FORMATION AFTER TREATMENT WITH SUBSTANCES WHICH LOWER SURFACE TENSION.

Exposure Minutes.	1 Per Cent. Amyl Alcohol.	2.5 Per Cent. Ethyl Acetate.	0.25 Per Cent. Ethyl Butyrate.	0.5 Per Cent. Ethyl Nitrate.	2.8 Per Cent. Ether.	'0.16 Per Cent.' Chloroform.	5 Per Cent. Acetonitrile.	Saturated Solution Phenyl Urethane.	0.2 Per Cent. Chloretone.	Emulsion[1] Toluol.	0.2 Per Cent. Saponin.
1	0		0	0	0		0		29		12
4	50		50	50	100		100		100		50
1	0	0	0	0	0	13	2	0	18	3	20
2	50	50	50	50	100	50	50	100	100	100	50
1	1	0	0	2	1	29	12	0	0	10	33
	50	50	50	50	100	50	50	100	100	100	50
2	12	4	22	14	6	e	e	0	0	e	
	25	50	100	100	100			100	100		
3	12	10	e	e	15	e	e	0	0	e	33
	25	50			100			100	100		50
4	31	13	e	e	22	e	e	0		e	18
	50	50			100			100			50
5		19[2]	e	e	18	e	e	1			20
		50			100			100			50
6	14	12 23[2]	e					2			
	25	50, 50						100			
7			e	e	15	e	e			e	19
					100						50
8		23						5			
		50						100			
10	3	16	e	e	5	e	e	9			
	25	50			100			100			
15								43			
								200			

All of the substances mentioned in the table produced a similar morphological change in the egg. All of them caused the vitelline membrane to become lifted away from the cytoplasm. The details of this membrane elevation were watched in a great many different experiments. The membrane first detached itself from the egg at a number of points around the periphery. As a result it appeared slightly thicker and for a minute or two it often

[1] This emulsion was made by stirring 1 c.c. of toluol with 3 c.c. or sea water.

[2] Eggs were shaken while counting. As mentioned above this gives a higher count.

showed small outbulgings which gave it a more or less crenate appearance. Very soon, the membrane lifted away with considerable rapidity. If success was to be obtained the egg had to be removed from the solution at just about the time the crenations appeared.

As in *Arbacia* when the vitelline membrane is lifted from the egg surface, a new membrane, no doubt a precipitation membrane, is immediately formed about the egg cytoplasm. Sometimes it is possible to cause this to become elevated also. Saponin produces the best results. When eggs are exposed for 40 minutes to 0.2 per cent. saponin they are found to be surrounded by two membranes. The outer one of these is quite far from the egg and it was thought at first that it might represent the outer edge of the jelly which had become visible. But this was shown not to be true, for two membranes could be produced about eggs from which the jelly had previously been shaken off.

I have used the eggs of *Cumingia* for a number of years and in some years I have found a tendency for a small per cent. of the eggs to mature without apparently any treatment. In the experiments recorded in this paper careful controls were always kept. In every case at least two hundred eggs of the untreated control were examined for polar body formation. Of the experiments cited in the table, in only one instance did the control show any maturation. In the control for the ethyl nitrate experiment one egg showed a polar body out of considerably over two hundred examined.

The table shows that eleven substances can produce polar body formation and this is in every case preceeded by membrane elevation. These eleven substances differ from each other very widely in chemical constitution. It is almost inconceivable that they should have any one chemical effect in common. Their action must be primarily physical. It is believed to involve a lowering of surface tension. The explanation which I have offered for membrane elevation in *Arbacia* applies equally well for *Cumingia*. For details of this explanation the reader is referred to my earlier papers.

Of course by choosing substances similar to those listed in the table numerous other successful reagents could no doubt be

discovered. Thus it is probable that benzol or xylol would behave as toluol, and that many other alcohols and esters could have been added to those given in the table. Such experiments would scarcely add material for the general argument.

On the other hand it might be thought that the eleven substances in the table represent only a few out of the many that I have tried. It perhaps not infrequently happens that experimenters suppress the record of their failures. But in these experiments practically every substance selected because of its effect on surface tension gave the expected result. There were only three exceptions and in two of these cases I performed only a single experiment with a single concentration of the reagent. Moreover in each of these three cases the reagent employed had some secondary effect on the egg. I shall consider each case in detail.

Ethyl Urethane.—3 per cent. ethyl urethane had practically no effect, although in longer exposures (5–7 minutes) a few eggs with polar bodies were observed. The reagent has some action on the jelly that I have not analyzed. Possibly it is a shrinkage effect. Two minutes after the egg entered the solution it was surrounded by queer looking bubbles. Fifteen minutes later the bubbles had disappeared and their place had apparently been taken by a zone of radiating lines.

Nitromethane.—I used a 5 per cent. solution of nitromethane and exposed the eggs to it for intervals of from $\frac{1}{2}$–10 minutes. No polar bodies were produced as a result of the treatment. Exposure for 4 minutes followed by transfer to normal sea-water resulted in rupture of the vitelline membrane and disintegration of the egg. The eggs left in the nitromethane solution showed a queer transformation. They lost their spherical shape and flattened out into discs resembling huge red blood corpuscles. In addition to this queer effect the reagent also appeared to have some action on the jelly, for some morphological changes were visible around the egg. The nature of these changes I did not stop to investigate.

Acetone.—Two concentrations of acetone were tried. 25 per cent. acetone did not produce membrane elevation when eggs were exposed 1–11 minutes. 50 per cent. acetone produced

membrane elevation, but not polar body formation. Of the eggs exposed ½ minute, 72 per cent. had widely elevated membranes. These eggs, however, could not produce polar bodies for they were thoroughly coagulated. This was shown by a viscosity test with the centrifuge.

Membrane Swelling.

If instead of being lifted off, the vitelline membrane is made to swell, much the same effect is produced on the egg. The increased fluidity of the vitelline membrane results in a lower surface tension.[1] Consequently it no longer exerts as great a pressure upon the egg contents. Thus maturation follows membrane swelling just as it follows membrane elevation.

In order to produce a swelling of the membrane the same reagents were used that were previously found to have been effective for the sea-urchin egg. Evidently the vitelline membranes of both *Cumingia* and *Arbacia* are similar, for they swell under the same conditions.

Sodium Iodide.—Eggs were exposed to 0.6 M sodium iodide. After 15 and 22½ minutes they were removed to sea-water in Stender dishes A and B respectively. Of the eggs in A, 14/100 showed polar bodies. The eggs in B formed no polar bodies. The sodium iodide solution caused membrane swelling.

Hydrochloric Acid.—Eggs were placed in 25 c.c. of sea-water plus 0.7 c.c. *n*/10 HCl. After exposures of ½, 1, 2, 3, 4, 5, 7, 10 minutes the eggs were removed from the acid solution and placed in ordinary sea-water in Stender dishes A–H respectively. In the acidified sea-water the egg membrane swelled and the surface of the egg became sticky. Often the eggs adhered to the bottom of the dish. Counts of eggs with polar bodies gave the following results:

A.....	½ minute exposure	6/50
B.....	1	5/50
C.....	1	10/50
D.....	3	4/50 (This count was made too early)
E.....	4	13/50
F.....	5	15/50
G.....	7	10/50
H.....	10	8/50

[1] Many biologists apparently do not understand that solids and pseudo-solids (*i.e.*, gels) exhibit surface tension. This tension is greater for a gel than for the corresponding sol. For references to literature on this subject consult Heilbrunn 15, footnote, p. 166.

The eggs which were allowed to remain in the acid sea-water also formed a few polar-bodies.

Potassium Hydroxide.—Eggs were placed in 40 c.c. of sea-water plus 1 c.c. *n*/10 KOH. In this solution membrane swelling occurred. The eggs formed polar bodies while in the alkaline medium. The first polar body observed was noted after 16 minutes exposure. After 33 minutes, a count gave 14/100 with polar bodies.

Potassium Cyanide.—Eggs were placed in a 0.04 per cent. KCN solution, made by diluting 5 c.c. of 2 per cent. KCN up to 250 c.c. with sea-water. During the experiment the cyanide was not allowed to evaporate, for it was kept in a tightly stoppered weighing-tube. After a 36 minute exposure a count showed 28/100 of the eggs with polar bodies. The potassium cyanide caused membrane swelling probably because of its alkaline reaction.

Hypertonic Sodium Chloride Solution.—In the experiments with acids and alkalies and with sodium iodide the solutions used were approximately isotonic with sea-water. In the course of some other work it was noticed that solutions made by adding $2\frac{1}{2}$ M NaCl to sea-water caused membrane swelling. Hence it was expected that these solutions would also cause polar body formation. Eggs were exposed for 7 minutes to a solution made by adding 5 c.c. of $2\frac{1}{2}$ M NaCl to 25 c.c. of sea-water. As a result 21/100 of the eggs formed polar bodies.

To sum up, in all of these experiments the swelling of the vitelline membrane was in every case followed by the throwing-off of polar bodies in a large percentage of the eggs. The controls of untreated eggs did not form polar bodies. In these experiments with reagents which cause membrane swelling there were no failures. No reagent could be discovered which would produce swelling of the vitelline membrane without at the same time causing the eggs to mature.

Rupture or Removal of the Membrane.

There is a third way in which the Cumingia egg may be freed from the binding pressure of its vitelline membrane. If the eggs are shaken vigorously, oftentimes a certain percentage of the

membranes will be shaken off. Apparently the membrane ruptures at the animal pole of the egg and owing to its elasticity it shrinks away toward the vegetal pole. In many cases its wrinkled remains can be found at this pole. No doubt after the membrane is shaken off a new precipitation membrane forms about the cytoplasm but this is very much less rigid than the original vitelline membrane. The results obtained from shaking *Cumingia* eggs are somewhat variable. The eggs must be shaken sufficiently to rupture or remove the membrane, but they must not be shaken too vigorously or too long. Too much shaking interferes with the mitotic processes underlying maturation and a smaller percentage of polar bodies results. This is in accordance with the observation of Wilson ('01) that shaking prevents cell division.

A number of shaking experiments were performed. In one of the best of these, the eggs were placed in a small 10 c.c. test tube and shaken vigorously by swinging the forearm from a vertical to a horizontal position. They were given 40 such swings in 10 seconds. When these shaken eggs were examined an hour and a half later it was found that 27/56 had polar bodies. Thus practically all of them had matured, for as pointed out in the first part of this paper, the counts represent minima. That the shaking process had actually resulted in a removal of the membrane could be demonstrated in three ways. In the first place the remains of the membrane could often be seen at the vegetal pole. Secondly, when the polar bodies formed they did not appear to be within a stiff membrane as in polar body formation after fertilization. Lastly some shaken eggs were placed in a drop of acetone, of these only 3 out of 50 showed membrane elevation. When normal unshaken eggs were similarly treated with acetone all of them showed an elevated membrane.

Finally there is still another method of freeing the egg from the restraint of its membrane. Although this method produces practically the same results as shaking, the procedure is very different. It was found that in diluted sea-water rupture of the vitelline membrane occurred and as was to be expected, maturation followed. Obviously in diluted sea-water the osmotic pres-

sure forces water to enter the eggs and the membrane bursts. In many instances the ruptured membrane could be seen at one side of the egg. Sometimes exovates were produced. Various dilutions were employed, from pure distilled water to a mixture of 1 part of distilled water to 2 parts of sea-water. In one experiment eggs were exposed to distilled water for 40, 60 and 80 seconds and then returned to sea-water. All three exposures were successful. The 40 second exposure was not counted, the 60 second exposure showed 51/100 polar bodies and the 80 second exposure 52/100 polar bodies. The controls were normal and over 200 eggs which were counted showed no signs of maturation. All the exposures showed some signs of cleavage. One egg reached a stage with about 16 cells. In another experiment eggs were placed into 30 c.c. of sea-water plus 15 c.c. of distilled water. Of these eggs 13/50 showed polar bodies.

The Significance of Cortical Change.

It has been shown that three types of cortical change can be produced in the *Cumingia* egg. All of these free the egg from the restraint of a stiff vitelline membrane. This release from restraint may then be thought of as the essential feature of cortical change and as the direct cause of maturation. When it occurs maturation follows; without it no maturation takes place.

It might be argued that all of the types of cortical change have some other common effect besides the one mentioned. Perhaps they all directly produce an increase of oxidations, which then causes maturation to follow. This would be a difficult relation to conceive of chemically. Moreover there is experimental evidence that maturation does not depend on an increase of oxidations.

The supporters of the oxidation theory of initiation of development have always held that dilute cyanide solutions check oxidations. Thus Loeb, '13, states on p. 26: "It has long been known that the oxidations in the cell can be prevented by the addition of a little potassium cyanide, even when oxygen is present. I have found that the addition of 0.5 c.c. of a 1/20 per cent. KCN solution to 50 c.c. of sea-water is sufficient to stop almost immediately the effect of the spermatozoön in the fertilized sea-urchin egg." Such a solution is 0.0005 per cent.

Some *Cumingia* eggs were placed in a 0.04 per cent. solution of potassium cyanide, five minutes after they had been fertilized. The solution was prepared by diluting 5 c.c. of 2 per cent. KCN up to 250 c.c. with sea-water. Solution and eggs were kept in a glass-stoppered weighing-tube to guard against evaporation of the cyanide. Under these conditions practically all of the fertilized eggs formed the first polar body. Actual count without turning over the eggs, showed 24/50.

On the other hand it might be thought that all types of cortical change produce an increase of permeability. In recent years various observers have claimed that the sea-urchin egg undergoes an increase in permeability either after fertilization or after artificial membrane elevation. These observers have endeavored to show: (1) An increased penetration of dyes, (2) a drop in electrical resistance, (3) a more rapid passage of water into or out of the cell.

When fertilized sea-urchin eggs are placed in dilute solutions of methylene blue, they stain more rapidly than do unfertilized eggs, according to Lyon and Shackell, '10. Runnström, '11, obtained similar results with methylene blue although not with neutral red. The experiments of Lyon and Shackell are frequently cited and are always taken to indicate an increased permeability after fertilization.[1] When unfertilized and fertilized *Cumingia* eggs are placed in dilute solutions of methylene blue or neutral red, the unfertilized eggs take up the dye just as rapidly as do the fertilized eggs.

Fertilized and unfertilized eggs were put into Syracuse dishes containing methylene blue solutions of various strengths. From time to time the eggs were examined over a light and over a dark background, and under the microscope. The color of faintly stained eggs can be much better observed with the naked eye than with the microscope. A more accurate method of determination would involve the use of a colorimeter but the method used was sufficient to show that no marked increase of permeability occurred. The dilutions of the dye were made up from a 0.5 per cent. solution of Grübler's "Methylenblau rectif. nach

[1] They might however indicate nothing more than an increased affinity for dyes on the part of the cytoplasm. Such changes in staining properties are common enough, especially after changes in the colloidal state.

Ehrlich." Five minutes after fertilization some fertilized eggs were placed in dishes A2 to E2 containing 0.1 per cent., 0.05 per cent., 0.025 per cent., 0.0125 per cent., 0.00625 per cent. methylene blue respectively. At the same time some unfertilized eggs were placed in dishes A1 to E1 containing similar concentrations of the dye. After ten minutes had elapsed, the eggs in A1 and A2 were navy blue, those in B1 and B2 pale blue, those in C1 and C2 scarcely colored and those in D1 and D2, E1 and E2 not colored at all. Obviously there was no difference between the two sets of eggs. After fifteen minutes eggs in A1 and A2 were navy blue, those in B1 and B2 light navy blue, those in C1 and C2 light blue, the unfertilized eggs in D1 were a very pale blue, the fertilized eggs in D2 uncolored, in E1 and E2 eggs were still uncolored. The eggs were observed at various times, but no marked changes could be observed. After an hour I thought I might be able to detect a slightly deeper color in the fertilized eggs in A2, B2, C2 than in the unfertilized eggs subjected to the same concentrations of dye in A1, B1, C1, but this was probably due to the fact that the fertilized eggs were slightly more numerous. If entrance of stain is a test of permeability, then certainly there is no sharp difference in the permeability of fertilized and unfertilized *Cumingia*. eggs. The concentrations of stain used in the above experiment were not injurious. Even in the most concentrated of the solutions used the eggs proceeded in their development and became motile larvæ. A similar experiment was tried with neutral red. A saturated solution of the stain and 1/2, 1/4, 1/8, 1/16 saturated solutions were used. In no case could any difference in permeability between fertilized and unfertilized eggs be noted.

McClendon, '10, and Gray, '16, have maintained that following fertilization there is a drop in the electrical resistance of the sea-urchin egg. They have interpreted this as indicating an increase of permeability, although of course various other explanations might be given. In the sea-urchin egg the normal process of cortical change is membrane elevation, which would interfere with the experiment. McClendon and Gray therefore were obliged either to wait until the eggs lost their power of undergoing membrane elevation or to so treat the eggs that they lost

this power, before they could make their measurements. In the *Cumingia* egg electrical measurements would be easier inasmuch as the normal cortical change is not membrane elevation.

But the following point should be noted. In any measurements of the electrical resistance of masses of egg cells, it is rather doubtful if one is measuring the resistance of the cells at all. If one conceives of a piled-up mass of spheres resting in a liquid, it is obvious after a moment's consideration that the liquid is broadly continuous from one side of the mass to the other. Thus water flows readily through a pile of shot. If now the spheres are poor conductors as compared to the liquid, and an electric current is sent through the mass, it will flow almost exclusively through the liquid. The resistance then depends on the size and shape of the interspaces between the spheres. In the case of egg cells this may vary in several ways. In the sea-urchin egg it is certain that after fertilization the interspaces between individual eggs in a mass are greater than those before fertilization, for it has been shown (cf. Heilbrunn, '15) that after fertilization the eggs offer much more resistance to compression and hence tend to preserve their spherical shape. Thus after fertilization one might expect the resistance of a mass of eggs to be lower even though the electric current did not pass through the eggs at all.

In the last few years R. S. Lillie ('16, '17) has shown that when fertilized or activated eggs are placed in hypotonic solutions, water enters them more rapidly than it does unfertilized eggs. Similarly in hypertonic solutions (R. S. Lillie, '18) water leaves the fertilized eggs more readily. This is due according to Lillie to an increased permeability of the plasma membrane to water. Lillie's reasoning is a bit difficult to follow. Originally he believed in an increased permeability of the membrane to salts and dissolved substances. This would of course decrease the speed of entrance of water from hypotonic solutions, or the speed of exit to hypertonic solutions, for it would decrease the osmotic pressure upon which the exit or entrance of the water depends. Osmotic pressure is, as everyone knows, dependent upon the impermeability of a membrane to dissolved substances. Increase in permeability to salts would therefore produce the opposite

effect from increased permeability to water. Apparently there is a dilemma.

As a matter of fact it appears to be rather far-fetched to assume a change in permeability to water, since we know the plasma membrane to be at all times permeable to it. R. Lillie's results can be much more simply explained on the basis of my conception of the plasma membrane (see Heilbrunn, '15). Normally before fertilization it is a more or less rigid structure and as such resists the entrance or exit of water from the cell. I showed by measurement ('15, pp. 155–158) that when the membrane was made less rigid as a result of membrane swelling then water left the cell more readily. After fertilization the plasma membrane either itself becomes less rigid, as when membrane swelling occurs, or it is replaced by a less rigid membrane as a result of membrane elevation. Hence water enters and leaves the cell more rapidly.

It is easy enough to decide between R. Lillie's interpretation and mine. If the difference is simply one of relative permeability to water, then in hypotonic or hypertonic solutions the water should enter or leave the fertilized eggs more rapidly, but the final equilibrium point should be the same for both fertilized and unfertilized eggs. However on the basis of my view, not only should the water enter and leave the eggs more rapidly, but the actual equilibrium state should be altered. In hypertonic solutions, more water should leave the fertilized eggs and in hypotonic solutions more water should enter them. In the case of hypotonic solutions Lillie's own figures seem to show that at equilibrium more water has entered the fertilized eggs than the unfertilized.[1]

[1] Lillie's measurements were made on the egg of the sea-urchin *Arbacia*. In this egg the presence of the elevated vitelline membrane or fertilization membrane introduces a complication. As is well known this membrane is a stiff structure. When fertilized sea-urchin eggs are subjected to hypotonic solutions they increase in size rapidly until they reach the elevated membrane. Then further increase in diameter is dependent on the power of the eggs to stretch or rupture the membrane. R. S. Lillie does not state which occurs. As a matter of fact these experiments on endosmosis were done in September when the *Arbacia* season is practically over. At this time the normal membrane elevation is difficult to obtain, and usually, unless the sperm concentration falls within certain very narrow limits, the membrane swells at fertilization. For eggs with swollen membranes the experiment is uncomplicated, as in *Cumingia*.

My experiments with *Cumingia* eggs show clearly that after fertilization not only do the eggs swell more rapidly in hypotonic solutions but their total imbitition of water is greater.

Five minutes after fertilization some eggs were placed in 25 c.c. sea-water plus 15 c.c. distilled water. At the same time some unfertilized eggs were placed in a similar solution. Both sets of eggs were measured after about 45 minutes. By this time the eggs had reached an osmotic equilibrium for a second set of measurements 45 minutes later showed no further change. The results are given in Table II.

TABLE II.

DIAMETERS OF FERTILIZED AND UNFERTILIZED EGGS IN 25 C.C. SEA-WATER + 15 C.C. DISTILLED WATER.

Average Diameter of 12 Unfertilized Eggs in Sea-water 62.65 μ.

Fertilized Eggs After 40–45 Min.	Fertilized Eggs After 85–90 Min.	Unfertilized Eggs After 47–55 Min.	Unfertilized Eggs After 92–100 Min.
μ	μ	μ	μ
70.4	69.2	66.9	67.5
69.2	69.8	66.9	67.5
73.9 × 66.9	68.7	66.9	66.9
71.0 × 68.1	69.2	68.1	66.4
69.8 × 66.9	69.2	66.9	68.1
69.8	71.5 × 63.6	68.1	67.5
69.2 × 68.7	71.5	67.5	66.9
68.1	70.4	66.9	67.5
71.0 × 67.5	69.2	66.4	68.7
70.4	70.4	68.1	68.1
72.7	69.8	68.7	67.5
70.4	70.4 × 68.1	68.1	66.9
69.2			
Average, 69.76	69.53	67.46	67.46

Measurements were made with a Spencer movable scale micrometer at a magnification of about 650 diameters. Not all the fertilized eggs remained spherical. For such eggs the longest and shortest diameters are recorded on the table. In computing the averages the mean of these two measurements was taken. It is obvious from the table that a greater amount of water entered the fertilized eggs. This can not be explained by assuming an increased permeability to water.

It should be noted that the vitelline membranes of the eggs in this experiment did not rupture as a result of being placed in the hypotonic solution. Such a rupture sometimes occurs in solutions of this strength.

In hypertonic solutions fertilized *Cumingia* eggs lose more water than do the unfertilized. The water does not merely leave the eggs more rapidly, more of it passes out and the osmotic equilibrium is different in the two cases. This is shown by an experiment in which fertilized and unfertilized eggs were placed in solutions prepared by adding 1 part of 2 *M* $MgSO_4$ to 2 parts of sea-water. Measurements were made as in the previous experiment. The results are given in Table III.

TABLE III.

DIAMETERS OF FERTILIZED AND UNFERTILIZED EGGS IN 30 C.C. 2M $MgSO_4$ + 60 C.C. SEA-WATER.

Average Diameter of 10 Unfertilized Eggs in Sea-water 61.74 μ.

Fertilized Eggs After 13–23 Min.	Fertilized Eggs After 163–172 Min.	Unfertilized Eggs After 27–33 Min.	Unfertilized Eggs After 143–161 Min.
μ	μ	μ	μ
57.7	51.9 × 58.3	59.4	60.6
53.7 × 58.9	57.7	60.0	60.0
56.5	43.3	59.4 (58.3)	61.2 (56.0)
57.7	42.7	61.7	58.9 (53.7)
57.1 × 58.3	57.7	60.0 (53.7)	60.6 (56.1)
57.1 (51.9)	56.5	59.4	58.9
56.5	57.1	59.4	59.4
59.5 × 54.2	53.7	60.6	57.7
56.0	53.1	61.2	58.9
55.4	49.0	59.4	59.4
	54.8	58.9 × 61.2	59.4
Average, 56.78	52.79	60.05	59.55

The above table requires a little explanation. Some of the eggs, usually the unfertilized ones, became slightly flattened at one pole while in the hypertonic solution. These eggs therefore had a slightly smaller volume than their diameter would indicate. In order to show the extent of the flattening a second measurement was taken from the flattened pole to the opposite pole of the egg. This second measurement is in every case shown in parentheses. Another point also needs explanation. The fertilized eggs measured in the second column had lost so much water in the hypertonic solution that their cytoplasm was coagulated and had in most cases begun to shrink away from the vitelline membrane. This shrinkage was most pronounced in the third and fourth eggs measured and this accounts for the very small size of these eggs.

The table shows clearly that more water leaves the fertilized than the unfertilized eggs. This can not be due to an increased permeability to water for an increase of this sort could produce no such effect. It must be due to a loss in the rigidity of the plasma membrane. In order to make this relation clear, I shall quote from p. 154 of the second paper of this series: "The plasma-membrane of the *Arbacia* egg is a protein gel. As such it possesses a certain degree of rigidity. Suppose a hypothetical system completely surrounded by an extremely rigid semi-permeable membrane. If such a system were placed in a concentrated solution no exosmosis could take place, for if the membrane were perfectly rigid, there could be no removal of solvent from the system without the production of a vacuum. But the membrane would be subjected to a considerable pressure which would tend to make it rearrange its particles in such a fashion that the volume enclosed within it might be lessened. Whereas an extremely rigid membrane would resist such forces one with only a certain degree of rigidity would yield (in the case of sufficient pressure) and exosmosis would be possible. Thus osmosis in an enclosed system depends to some extent at least on the rigidity of the confining membrane. These conclusions apply in some measure to the sea-urchin egg, for the vitelline membrane possesses a slight degree of rigidity." They apply even more directly to the Cumingia egg, for its vitelline membrane, which is also its plasma membrane, is stiffer than that of *Arbacia*. Any loss in the rigidity of this membrane favors either endosmosis or exosmosis. That is why the fertilized eggs of *Cumingia* take up more water from hypotonic solutions and lose more water to hypertonic sloutions than do the unfertilized eggs.

Osmotic change in unfertilized eggs is fairly rapid and no one can deny that the plasma membrane is permeable to water. If then R. S. Lillie's measurements are correct and water enters fertilized eggs more rapidly than unfertilized eggs in hypotonic solutions and leaves them more rapidly in hypertonic solutions than it seems certain that the egg plasma membrane has not markedly increased its permeability to dissolved substances as a result of fertilization. For such an increase in permeability,

by diminishing the osmotic pressure, would slow osmotic interchange and if sufficiently great, would prevent it altogether.

All these points show clearly that the permeability theory of fertilization and artificial parthenogenesis rests on rather doubtful evidence. In *Cumingia* certainly, there are no facts which support it.

Discussion.

Bataillon ('12) in discussing the relations between artificial parthenogenesis in amphibia and sea-urchins, states, "Il n'y a pas une parthénogenèse expérimentale des Oursins et une des Amphibiens. Ce sont des matériaux différents chez lesquels le rythme des cinèses est suspendu et peut être rétabli. S'il y a une Biologie générale, les conditions de l'arrêt ont quelque chose de commun, et les conditions de la mise en branle doivent être comparable." Presumably this is true. The essential factors underlying stimulation to development are very probably alike for every sort of artificial parthenogenesis. It is certain, however, that the subsidiary features of the process are different in each case. It is necessary therefore to study each egg individually, to determine exactly its physical make-up, and to attempt to discover what changes are significant in producing an initiation of development. This is what I tried to do in the case of *Arbacia*.

F. R. Lillie ('19) in referring to my explanation of the process of cortical change in *Arbacia* points out that this explanation "can hardly apply to other cases where the cortical changes present a different morphological form." This is true and I never intended that it should. As a matter of fact, I clearly recognized that even in the one egg there were two distinct types of cortical change which might be produced by spermatozoa. The explanation which F. R. Lillie cites was only advanced to cover one of them. At the same time I offered a different explanation for the other.

F. R. Lillie also objects to my considering cortical change as "a mere epiphenomenon . . . the phenomenon of the primary cortical change is too general to be treated in this fashion and its character in different animal groups is too varied for it to be a mere phenomenon of decrease of surface tension." I must point

out that I have always realized the importance of cortical change. On page 183 of my 1915 paper I stated in a section devoted to the significance of cortical change, that "cortical change, whether it be membrane swelling or elevation, always results in the removal of this obstacle [*i.e.*, a stiff membrane]. The vitelline membrane is either rendered soft by swelling or it is lifted away from the egg surface and its place taken by the no doubt less rigid hyaline layer." I showed moreover that "at least two processes which play a part in normal development would be greatly hindered if some kind of cortical change did not occur."

My results with *Cumingia* fully bear out this point of view. Cortical change in *Cumingia* may take the form of a membrane elevation dependent on a sharp decrease in surface tension. It may more simply be just a membrane swelling. In both cases the result is the same. The egg is freed from an obstacle which impedes development. This in *Cumingia* as in *Arbacia* is the restraining influence of a stiff vitelline membrane. In both eggs the same forces are involved in cortical change. The essential features of the process and the effect on further development are as closely alike as they could possibly be in the two cases.

It should be noted that cortical change in *Cumingia* is not ordinarily followed by segmentation. As in *Arbacia* cell-division in *Cumingia* is preceded by a sharp increase in the viscosity of the cytoplasm. This can be demonstrated by tests with the centrifuge. A number of such tests were made and the relation established beyond a doubt.

Summary.

1. The *Cumingia* egg is surrounded by a stiff vitelline membrane which tightly encloses the fluid cytoplasm.

2. A release from the restraint of this membrane is followed by maturation.

3. Such a release from restraint can be accomplished in three ways; by membrane elevation, by membrane swelling, or by the removal or rupture of the membrane.

4. Substances which themselves have low surface tension produce a lowered surface tension of the membrane and this results in its elevation from the egg surface.

5. Acids, alkalies, and certain salt solutions cause the vitelline membrane to swell.

6. The membrane may be removed from the eggs by shaking, or it may be caused to rupture by immersion in dilute sea-water.

7. All of the above mentioned treatments produce polar-body formation. All of them free the egg from restraint.

8. Maturation in *Cumingia* is not dependent on an increase in oxidations.

9. Cortical change in *Cumingia* produces no increase in permeability either to dissolved substances or to water.

10. The essential features of cortical change in *Cumingia* are the same as those previously shown for Arbacia.

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